

Acute alcohol and chronic drinking bidirectionally regulate the excitability of prefrontal cortex vasoactive intestinal peptide interneurons

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ABSTRACT

The prefrontal cortex (PFC) regulates drinking behaviors and affective changes following chronic alcohol use. PFC activity is dynamically modulated by local inhibitory interneurons (INs), which can be divided into non-overlapping groups with distinct functional roles. Within deeper layers of neocortex, INs that express either parvalbumin or somatostatin directly inhibit pyramidal cells. By contrast, the plurality of all remaining INs express vasoactive intestinal peptide (VIP), reside within superficial layers, and preferentially target other types of INs. While recent studies have described adaptations to PFC parvalbumin-INs and somatostatin-INs in alcohol use models, whether ethanol or drinking affect the physiology of PFC VIP-INs has not been reported. To address this gap, we used genetically engineered female and male mice to target VIP-INs in layers 1–3 of prelimbic PFC for whole-cell patch-clamp electrophysiology. We found that ethanol (20 mM, ~0.09 BEC/90 mg/dL) application to PFC brain slices enhances VIP-IN excitability. We next examined effects following chronic drinking by providing mice with 4 weeks of intermittent access (IA) ethanol two-bottle choice in the home cage. In these studies, VIP-INs from female and male IA ethanol mice displayed reduced excitability relative to cells from water-only controls. Finally, we assessed whether these effects continue into abstinence. After 7–13 days without ethanol, the hypo-excitability of VIP-INs from male IA ethanol mice persisted, whereas cells from female IA ethanol mice were not different from their controls. Together, these findings illustrate that acute ethanol enhances VIP-IN excitability and suggest these cells undergo pronounced homeostatic changes following long-term drinking.

1. Introduction

Breakthrough treatments are needed to meet the ongoing public health challenges related to maladaptive drinking and alcohol use disorder (AUD) (Grant et al., 2017; Whiteford et al., 2013). Developing new neuropharmacological treatments necessitates a deeper understanding of the mechanisms through which ethanol alters brain circuit function. Studies in humans and in animal models suggest that difficulty in moderating drinking and affective disruptions during abstinence are each associated with the prefrontal cortex (PFC) (Blaine et al., 2020; Koob, 2014). In addition, differences in PFC function, at the population level, have been related to heavy drinking or AUD diagnosis (Medina et al., 2008; Sorg et al., 2012; Wang et al., 2016). A better understanding

of how specific PFC cell types are affected by ethanol and drinking should inform new avenues for treatment development.

PFC pyramidal cells regulate drinking through long-range glutamatergic projections to subcortical structures (Klenowski, 2018; Seif et al., 2013; Siciliano et al., 2019). Nonetheless, local inhibitory interneurons (INs) orchestrate the activity of pyramidal cells. At the highest level, neocortical interneurons can be subclassified by mutually exclusive expression of parvalbumin (PV), somatostatin (SST), or (in rodents) the 5HT₃ serotonin receptor (Lee et al., 2010; Rudy et al., 2011). PV-INs and SST-INs are related cell types that arise from the medial ganglionic evidence during development and preferentially reside in deeper cortical layers. By contrast, 5HT₃-INs arise from the caudal ganglionic evidence and represent the majority of IN subtypes

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within the superficial cortical layers. Accumulating evidence indicates that acute ethanol, voluntary drinking, and dependence all alter the activity of PFC PV-INs and SST-INs (Dao et al., 2020, 2021; Ferranti et al., 2022; Fish and Joffe, 2022; Hughes et al., 2020; Joffe et al., 2020b; Li et al., 2021; Trantham-Davidson et al., 2014, 2017). Considering these two IN subtypes regulate PFC circuit function through distinct subcellular targets and mechanisms, it is unsurprising that prior studies performing head-to-head comparisons each observed contrasting adaptations to PV-INs and SST-INs in alcohol use models (Hughes et al., 2020; Joffe et al., 2020b). In addition, other specialized IN subtypes most likely undergo distinct adaptations in response to ethanol. At this point, however, very little is known regarding how ethanol affects the physiology or function of any subclass of 5HT₃-IN.

Vasoactive intestinal peptide (VIP) is exclusively expressed by GABAergic neurons in neocortex (Pronneke et al., 2015; Tasic et al., 2018). VIP-INs represent the plurality of 5HT₃-INs (approximately 40% of 5HT₃-INs and 10% of all INs) and reside primarily within superficial layers of neocortex (Lee et al., 2010). Most VIP-INs are bipolar cells that co-express calretinin (Cauli et al., 2014; Kawaguchi and Kubota, 1997) and display extremely high membrane resistance (R_m), rendering them highly responsive to stimulation (Anastasiades et al., 2018; Lee et al., 2013; Pronneke et al., 2015; Schuman et al., 2019). Unique among IN subtypes, VIP-INs display minimal innervation of pyramidal cells and instead preferentially target SST-INs (Anastasiades et al., 2018; Fu et al., 2015; Lee et al., 2013; Melchitzky and Lewis, 2008; Pfeffer et al., 2013; Pi et al., 2013). Through this disinhibitory motif, VIP-INs are activated by subcortical nuclei to decrease spontaneous SST-IN activity, increase the gain through cortical circuits, and ultimately facilitate pyramidal cell activity (Audette et al., 2018; Lee et al., 2013; Pi et al., 2013; Williams and Holtmaat, 2019). VIP-INs are therefore well-positioned to regulate the effects of drugs and other salient experiences; nonetheless, we are not aware of any published studies that have reported how ethanol or drinking affect the physiology of PFC VIP-INs.

To address this knowledge gap, we aimed to test whether alcohol alters the physiology of PFC VIP-INs. Using targeted whole-cell patch-clamp techniques, we discovered that acute application of a modest concentration of ethanol (20 mM/0.09 BEC/90 mg/dL) increases the excitability of PFC VIP-INs in mice. Next, to ask whether chronic drinking induces similar or related adaptations to VIP-INs, we provided mice with intermittent access (IA) ethanol. In contrast to the acute application studies, we found that VIP-INs from IA ethanol mice displayed decreased spike-firing relative to cells from water-only controls, suggesting that chronic drinking can induce a homeostatic reduction in VIP-IN excitability. Finally, we found that VIP-INs underwent sex-dependent changes during 7–13 days of abstinence: VIP-INs from male IA ethanol mice exhibited persistent hypo-excitability relative to controls, whereas VIP-INs from female IA ethanol mice were not different from respective controls. Together, these studies describe adaptations to PFC VIP-INs following acute ethanol, chronic drinking, and abstinence, suggesting that neocortical disinhibitory microcircuits contribute to acute and chronic adaptations related to alcohol use.

2. Material and methods

2.1. Mice

Mice were housed on a standard 12-h light cycle (on at 6:00 a.m.). Mice were bred on a congenic C57BL6/J background to express tdTomato in PFC VIP-INs. Female VIP-Cre mice (Madisen et al., 2010) (*Vip^{cre}/Z^h/J*, Jackson Laboratories, Stock No: 010908) were crossed with male “Ai9” mice (Taniguchi et al., 2011) (*B6.Cg-t(Rosa)26Sor^{tm9}(CAG-tdTomato)Hze/J*, Jackson Laboratories, Stock No: 007909). We classified mice as female or male based only on external genitalia, and all mice were used for experiments. Comparable trends for the acute effects of ethanol on VIP-IN physiology emerged in neurons from both female mice and male mice, so data were pooled for the primary analyses. Given

sex differences in voluntary drinking in rodents, all analyses for IA ethanol experiments accounted for sex as a separate variable. For the IA ethanol experiments, mice were group-housed (2–5 per cage) in clear polysulfone individually ventilated cages until IA ethanol experiments began, no earlier than 6-weeks of age (range P48–P79), after which they were individually housed. All cohorts consisted of sex-matched littermates.

2.2. Electrophysiology

We used whole-cell patch-clamp electrophysiology to assess VIP-IN membrane and synaptic physiology. As in previous studies (Joffe et al., 2020a, 2022), we rapidly prepared 300- μ m coronal brain slices containing prelimbic PFC using an NMDG-based cutting and recovery solution (in mM): 93 NMDG, 2.5 KCl, 0.5 CaCl₂, 10 MgSO₄, 1.2 NaH₂PO₄, 30 NaHCO₃, 25 glucose, 20 HEPES, 5 Na-ascorbate, and 3 Na-pyruvate; pH adjusted to 7.3 with HCl, 300–310 mOsm. All solutions were bubbled with 95% O₂/5% CO₂. Slices recovered in standard artificial cerebrospinal fluid (ACSF) at 20–24 °C for 1–6 h before recordings. ACSF contained (in mM): 119 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 1 NaH₂PO₄, 26 NaHCO₃, and 11 glucose; 292–296 mOsm. Slices were transferred to a recording chamber perfused with 30–32 °C at 2 mL/min under an upright microscope. We targeted VIP-INs in layers 1–3 prelimbic PFC via red, tdTomato fluorescence and patched cells using pulled glass pipettes (5–7 M Ω) filled with a potassium-based internal solution (in mM): 125 K-gluconate, 5 NaCl, 0.2 EGTA, 10 HEPES, 4 MgATP, 0.3 Na₂GTP, 10 Tris-phosphocreatine; pH adjusted to 7.3 with KOH, 292–296 mOsm.

Cells were dialyzed with internal solution for 5 min before undergoing a series of 1-sec current injections [–30 to +150, Δ 5 pA] to assess VIP-IN membrane properties. We examined several physiological properties, including: (1) resting membrane potential (V_m), (2) membrane resistance (R_m), (3) hyperpolarization sag, (4) rheobase, and (5) spike firing input-output curve. R_m was calculated as the best-fit slope from three hyperpolarizing current injections [–5, –10, –15 pA]. The hyperpolarization sag ratio was quantified as the difference between the peak hyperpolarization and the steady-state hyperpolarization normalized to the difference between the steady-state hyperpolarization and the resting potential. The sag ratio was calculated as the average value across three consecutive current injections [–20, –25, –30 pA]. The rheobase and spike-firing input-output curves were collected during and after depolarizing current injections. Rheobase was determined as the minimum current step that initiated at least one action potential. Action potentials were identified using a threshold search event detector and the number of spikes per current step were reported. Cells that spontaneously fired action potentials were discarded. After current-clamp recordings, cells were voltage-clamped at –80 mV to electrically isolate spontaneous excitatory postsynaptic currents (sEPSCs) (Joffe et al., 2020b). sEPSCs were identified using a template search event detector.

For ethanol application studies, slices were incubated in 20 mM ethanol for at least 1 h before, and throughout, all recordings. Preliminary studies found minimal effect of 5-min ethanol applications. Control recordings in standard ACSF were always recorded from the same animals on the same recording days in a double-alternating, counterbalanced manner. In some studies, we prepared acute brain slices after 4 weeks of voluntary drinking. During the light cycle following a night of ethanol access, mice were removed from their home cages for 1–2 h, transferred from the colony room to the laboratory in a clean, novel bucket, and sacrificed under deep isoflurane anesthesia. In some experiments, slices were prepared after 7–13 days following forced abstinence. We recorded from 3 to 6 cells per animal and did not find evidence for any correlations between an animal's ethanol consumption and any physiological parameter. The experimenter was blinded to condition for all studies following chronic drinking.

2.3. IA ethanol

As in our previous studies (Joffe et al., 2020b, 2021), we modeled high levels of volitional alcohol drinking by an intermittent access (IA) schedule. For three 24-h periods per week, ethanol was provided in home cages. Ethanol was removed for 24–48 h between each exposure, and mice had constant access to food and water throughout all experiments. Ethanol was diluted from 95% undenatured ethyl alcohol (Decon Labs, Incorporated). Ethanol and water were delivered by conical tubes capped with rubber stoppers (Fisherbrand 14–135H, Fisher Scientific) and open tip straight tubes (OT100, Ancare). Fluid consumed was calculated by measuring tube weight before and after each session, subtracting the average drip (0.2 g) from all values. Ethanol was generally provided and removed 3–4 h prior to the transition to the dark cycle. The ethanol concentration increased over the first three sessions [3, 6, 10%] after which 20% ethanol was provided for the remainder of the study.

2.4. Statistics

The number of cells and mice are denoted by “n” and “N”, respectively, for each experiment. ClampFit 11.2 (Molecular Devices) was used to for primary analysis and subsequent analyses were performed in Excel (Microsoft) and Prism 9.4 (GraphPad). Data are presented as mean $\pm$ standard error or as box plots. Data were analyzed using two-tailed Student’s t-test, or Mann-Whitney test if a dataset failed a Shapiro-Wilk normality test. Two-way ANOVA or mixed-effects analysis with Bonferonni post hoc comparisons were also used to detect significance.

3. Results

3.1. Minimal sex differences in membrane physiology of PFC VIP-INS

Within neocortex, VIP is selectively expressed by disinhibitory interneurons enriched in superficial layers (Fig. 1a). To target these cells in a high-throughput manner, we bred mice to express red fluorescent protein in PFC VIP-INS by crossing homozygous VIP-Cre female mice (Madisen et al., 2010) with male mice harboring a Cre-dependent tdTomato locus (Taniguchi et al., 2011). All breeders and experimental mice were on a congenic C57BL/6J background. We made acute brain slices from adult (8–20-week) mice and performed whole-cell patch-clamp electrophysiology experiments to examine VIP-IN membrane physiology parameters (Fig. 1b). As reported in previous studies (Anastasiades et al., 2018; Goff and Goldberg, 2019; Kawaguchi, 1997; Porter et al., 1998; Pronneke et al., 2015), we found PFC VIP-INS to be highly excitable cells with considerable heterogeneity in spike-firing patterns and subthreshold properties.

To our knowledge, no published studies have systematically assessed whether VIP-INS display sex differences in physiology. We therefore performed a head-to-head comparison of several membrane properties following negative and positive current injections. Overall, we found no evidence for sex differences in membrane properties of PFC VIP-INS. VIP-INS from female and male mice displayed comparable current-evoked action potentials (Fig. 1c). VIP-IN resting membrane potential (V_m) is comparable to other cell types within PFC, and no differences related to sex were detected (Fig. 1d). By contrast, relative to pyramidal cells and most other IN subtypes, VIP-INS displayed extremely low rheobase (Fig. 1e) – i.e., the minimum current needed to elicit spike-firing. Additionally, VIP-INS exhibit extremely high membrane resistance (R_m) (Fig. 1f), an overall measurement of excitability that is inversely proportional to the number of functional cell-surface ion channels. No sex differences in VIP-IN rheobase or membrane resistance were detected. Finally, we assessed hyperpolarization sag (Fig. 1g), a measurement of hyperpolarization- and cyclic nucleotide-gated (HCN) channels (Salling et al., 2018; Shah, 2014), which can regulate how certain subtypes of hippocampal INs respond to acute ethanol (Yan

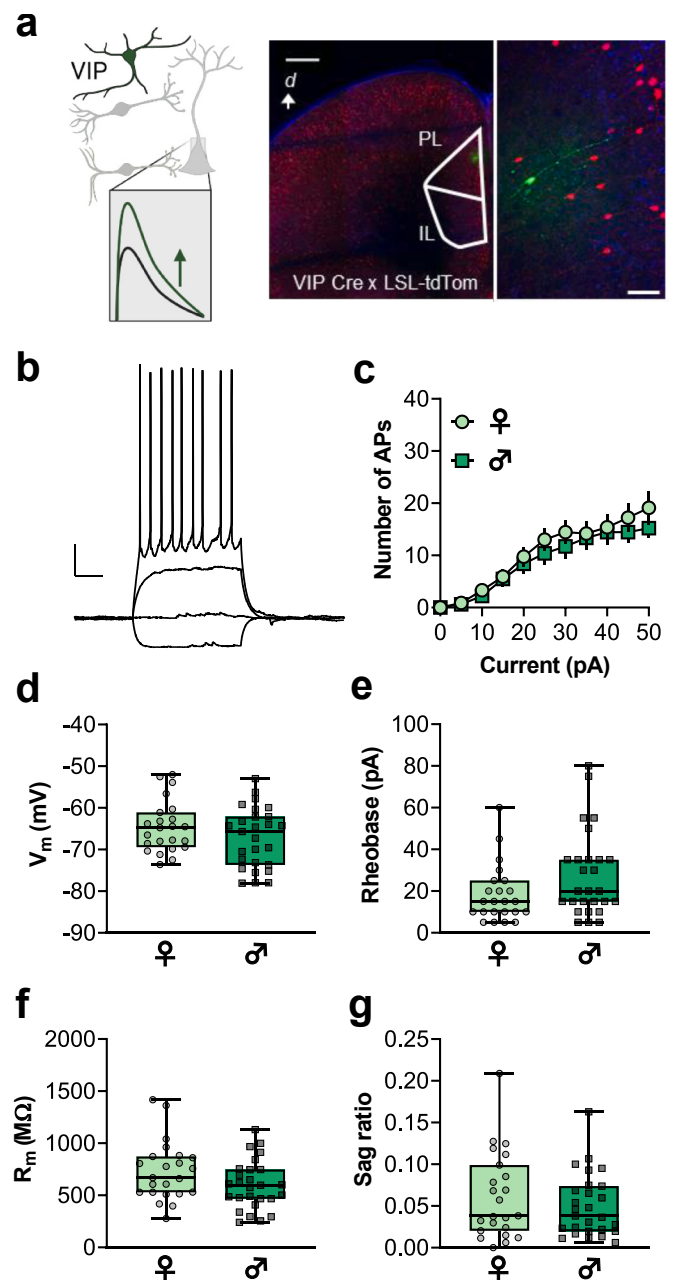


Fig. 1. Minimal sex differences in membrane physiology of PFC vasoactive intestinal peptide inhibitory interneurons (VIP-INS). (a) VIP-INS inhibit other IN subtypes to enhance excitatory postsynaptic potentials in pyramidal cells. VIP-Cre mice were crossed with Ai9 mice (Rosa26-Lox-STOP-Lox-tdTomato) to generate mice expressing fluorescent proteins in VIP-INS. A representative image of a coronal brain slices depicts a representative cell filled with Alexa Fluor 488 green dye. d, dorsal; IL, infralimbic; PL, prelimbic. Scale bars 500 μ m and 50 μ m. (b) Representative VIP-IN current-clamp recording in response to current injections (–10 to +20 pA, in 10 pA steps). Scale bars 20 mV and 250 ms. (c) Current-evoked action potentials (APs) were comparable in VIP-INS from female (♀) and male (♂) mice. (d) No difference in resting membrane potential (V_m) between ♀ and ♂ mice. (e) Mouse sex did not affect VIP-IN rheobase. (f) VIP-IN membrane resistance (R_m) was not different between ♀ and ♂ mice. (g) Hyperpolarization sag ratio was similar in VIP-INS from ♀ and ♂ mice. n/N = 23–27 cells from 5 to 7 mice per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2009, 2010). Hyperpolarization sag was similar in VIP-INs from female and male mice. Taken together, these data indicate that VIP-INs are highly excitable cells without obvious basal sex differences in membrane physiology.

3.2. Acute ethanol application enhances excitability of PFC VIP-INs

To probe whether ethanol directly can affect VIP-IN membrane physiology, we incubated acute PFC brain slices in 20 mM ethanol [~ 0.09 BEC (90 mg/dL), comparable to peaks concentrations attained during IA ethanol (Salling et al., 2018)] for 1–5 h before recording from VIP-INs (Fig. 2a). Comparable effects were observed in cells from female and male mice (Fig. S1), so data were pooled for the primary analysis. VIP-INs treated with ethanol fired more action potentials in response to positive current injections than ACSF control cells (Fig. 2b and c). Acute ethanol did not alter VIP-IN resting membrane potential (Fig. 2d); however, ethanol reduced rheobase (Fig. 2e) and enhanced membrane resistance (Fig. 2f) relative to matched control ACSF cells. Finally, hyperpolarization sag was not affected by ethanol application (Fig. 2g), suggesting that 20 mM ethanol does not acutely affect HCN channel function in VIP-INs in brain slices. The length of time of ethanol (or control ACSF) incubation was not related to any physiological parameter (Fig. S2). Taken together, these findings suggest that ethanol can directly act on VIP-INs to enhance their excitability.

3.3. Chronic voluntary drinking decreases excitability of PFC VIP-INs

The previous findings indicated that acute ethanol administration can alter VIP-IN excitability, so we next asked whether long-term drinking induces similar or related effects. We examined VIP-IN membrane physiology following intermittent access (IA) ethanol, a model that we and others have used to facilitate moderate-to-high levels of voluntary drinking in mice (Crabbe et al., 2012; Joffe et al., 2020b, 2021; Salling et al., 2018). For alternating 24-h periods, we provided VIP-tdTomato mice with free access to ethanol in their home cages (Fig. 3a). Female C57BL/6J mice generally drink more ethanol than male counterparts (Becker and Lopez, 2004; Finn et al., 2018; Joffe et al., 2020b; Jury et al., 2017). In the cohort used for these experiments, we observed slightly greater drinking in female mice, but the effect did not reach statistical significance (Fig. S3).

The day after the last drinking session, we sacrificed mice for brain slice electrophysiology and recorded from PFC VIP-INs. As with the acute ethanol studies, we examined VIP-IN membrane properties through a series of negative and positive current injections (Fig. 3b). While we previously found that acute ethanol enhances VIP-IN excitability, we observed the opposite effect following chronic drinking: VIP-INs from IA ethanol mice displayed decreased current-evoked spike-firing relative to matched cells from water-drinking controls (Fig. 3c and d). The resting membrane potential was not affected by IA ethanol or sex (Fig. 3e), suggesting that changes in spike-firing likely stem from a voltage-gated conductance. Consistent with the overall decrease in current-evoked spiking, drinking increased the minimum current required to elicit action potential firing in VIP-INs, as quantified by a greater rheobase in IA ethanol cells relative to controls (Fig. 3f). IA ethanol did not affect membrane resistance, although VIP-INs from male mice displayed reduced membrane resistance relative to cells from female mice in this cohort (Fig. 3g). Finally, no effect of drinking or sex was found on hyperpolarization sag (Fig. 3h). In addition, VIP-IN rheobase or R_m did not correlate with ethanol intake per mouse, when analyzed in either sex independently or all animals combined (Fig. S4). Taken together, these studies illustrate that chronic drinking generates opposing adaptations to VIP-INs relative to acute ethanol application.

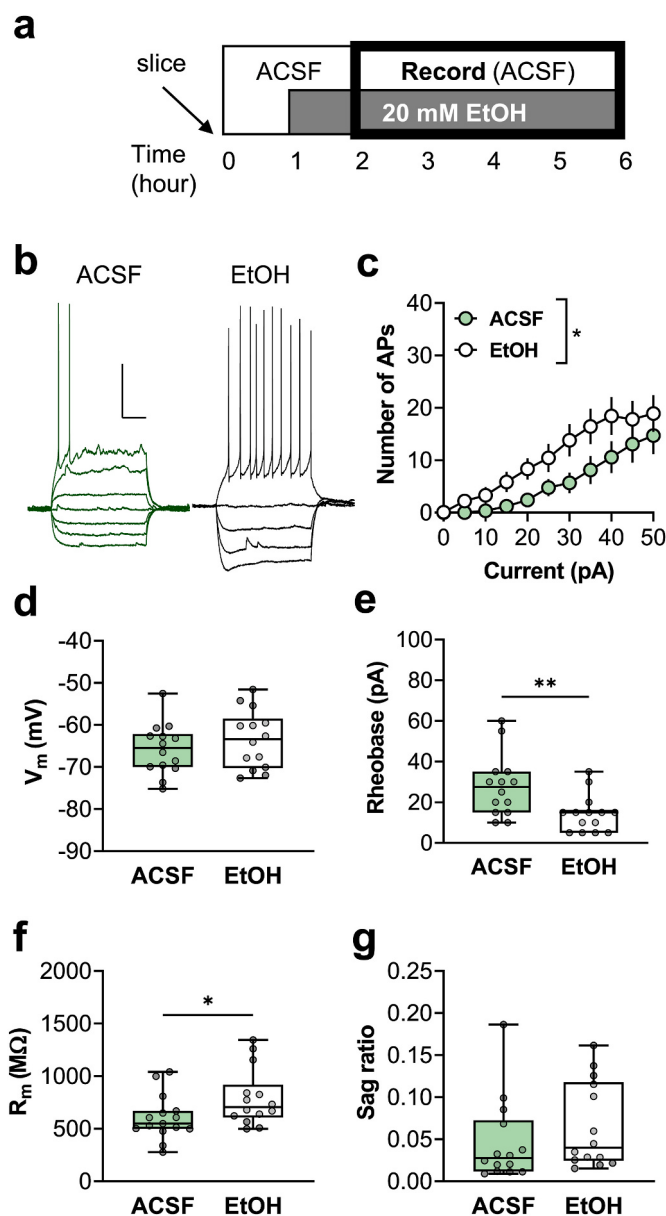


Fig. 2. Acute ethanol application enhances excitability of PFC VIP-INs. (a) Experimental schematic. All slices recovered in ACSF for 1 h, at which point half of the slices per mouse were transferred to ACSF supplemented with 20 mM ethanol. Recordings from ACSF or ethanol slices were then made up to 6 h after sacrifice. (b) Representative VIP-IN current-clamp recordings from control (left, green) and 20 mM ethanol (right, black) slices. Traces depict responses to incremented positive current injections (-30 to $+30/10$ pA, in 10 pA steps). Scale bars 20 mV and 250 ms. (c) Acute ethanol enhanced VIP-IN current-evoked spiking (Two-way RM mixed-effects analysis: $F_{1,26} = 6.5$, $^*p < 0.05$ main effect of IA ethanol). (d) VIP-IN V_m was not affected by acute ethanol. (e) Ethanol application decreased VIP-IN rheobase (Mann-Whitney test: $U = 41.5$, $^{**}p < 0.01$). (f) Ethanol increased VIP-IN R_m (Mann-Whitney test: $U = 44.5$, $^*p < 0.05$). (g) VIP-IN hyperpolarization sag was not affected by acute ethanol. $n/N = 14$ cells from 7 mice (4♀/3♂) per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

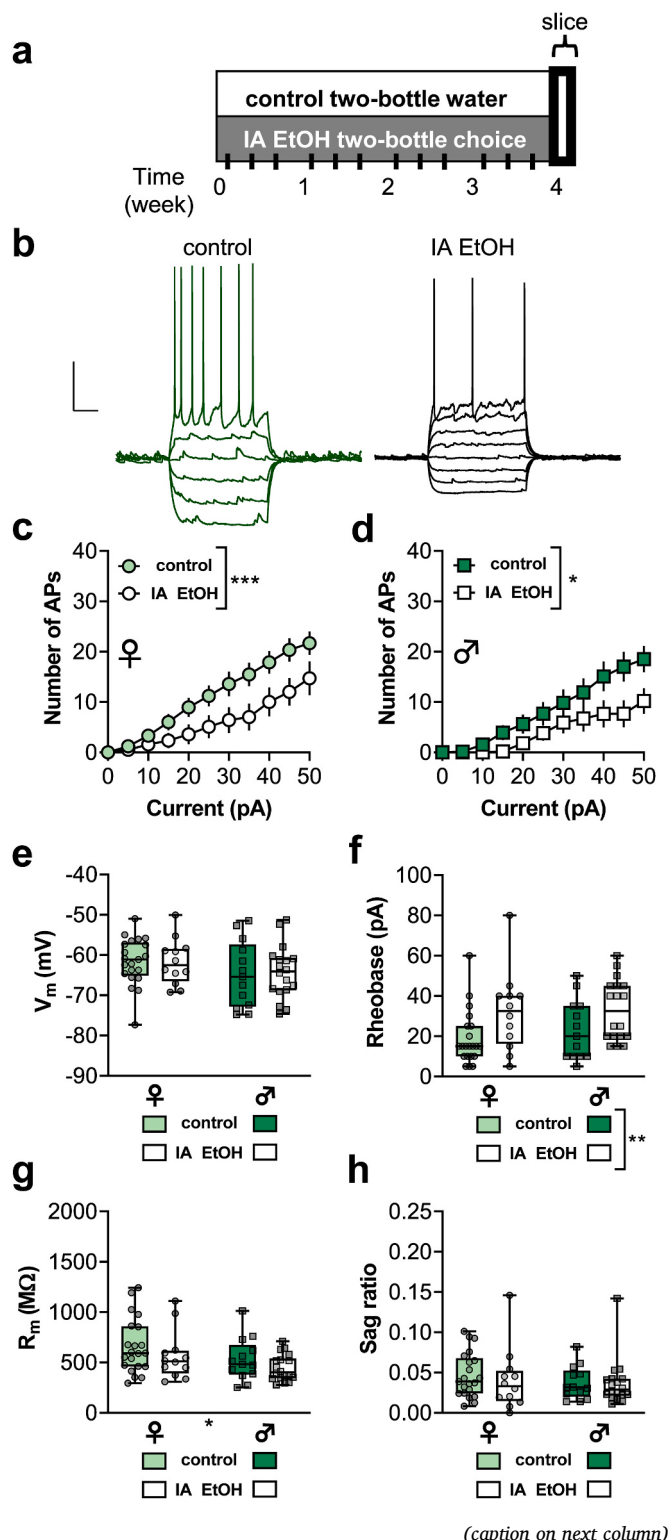


Fig. 3. Chronic voluntary drinking decreases excitability of PFC VIP-INs. (a) Experimental schematic. VIP-tdTomato mice underwent 4 weeks of intermittent access (IA) ethanol two-bottle choice, or control two-bottle water, home cage drinking. Slices were prepared the day after the last drinking session. (b) Representative VIP-IN current-clamp recordings from control (left, green) and IA ethanol mice (right, black). Traces depict responses to incremented positive current injections (-30 to +20/40 pA, in 10 pA steps). Scale bars 20 mV and 250 ms. (c/d) IA ethanol decreased current-evoked spiking in VIP-INs in both ♀ mice (Two-way RM mixed-effects analysis: $F_{10,288} = 3.2$, ***: $p < 0.001$ current $\times$ IA ethanol interaction) and ♂ mice (Two-way RM mixed-effects analysis: $F_{10,213} = 2.3$, *: $p < 0.05$ current $\times$ IA ethanol interaction). (e) VIP-IN V_m was not affected by IA ethanol. (f) IA ethanol increased VIP-IN rheobase (Two-way ANOVA: $F_{1,60} = 8.5$, **: $p < 0.01$ main effect of IA ethanol). (g) IA ethanol did not affect VIP-IN R_m , but VIP R_m was lower in ♂ mice relative to ♀ mice in this cohort (Two-way ANOVA: $F_{1,60} = 4.8$, *: $p < 0.05$ main effect of sex). (h) No effect of IA ethanol on hyperpolarization sag. $n/N = 13$ –20 cells from 3 to 5 mice per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Hypo-excitability of VIP-INs persists in male mice but not female mice

Several affective behavioral changes develop, and some behavioral changes wane, over the course of abstinence from voluntary drinking (Bloch et al., 2022; Holleran and Winder, 2017). We therefore aimed to understand whether drinking-associated changes to VIP-IN physiology persist into abstinence. To address this question, we provided a new cohort of mice with IA ethanol for 4 weeks, after which we removed all access to ethanol for 1–2 weeks before sacrificing animals for electrophysiology (Fig. 4a). In contrast to the studies performed without abstinence, we observed no differences in current-evoked spike-firing (Fig. 4b), or any other physiological parameter, in VIP-INs from female mice. VIP-INs from abstinent IA ethanol male mice, however, displayed decreased current-evoked spiking relative to control cells (Fig. 4c). Furthermore, VIP-INs from abstinent IA ethanol male mice had hyperpolarized resting membrane potentials relative to controls (Fig. 4d). Similar to previous studies at the early timepoint, VIP-INs from male mice in abstinence displayed greater rheobase (Fig. 4e) and decreased membrane resistance (Fig. 4f) than control cells. Neither VIP-IN rheobase nor R_m correlated with the duration of abstinence from IA ethanol (Fig. S4). Finally, we assessed hyperpolarization sag potentials. VIP-INs from abstinent male mice displayed reduced hyperpolarization sag than controls (Fig. 4g), consistent with a potential reduction in HCN channel function. Overall, these findings suggest that VIP-INs from female mice may recover faster from the effects of chronic drinking when compared to male experimental counterparts. Furthermore, the changes in resting potential and hyperpolarization sag both suggest that additional adaptations in VIP-INs HCN channel function may develop during abstinence in male mice.

3.5. Minimal effect of acute ethanol, IA ethanol, or abstinence on excitatory drive onto VIP-INs

In addition to intrinsic physiology, changes in synaptic strength can exert a major impact on a cell's integration within its micro- and macro-neuronal circuits. Previous studies from our lab and others have described several changes to excitatory drive onto other IN subtypes in PFC (Dao et al., 2021; Ferranti et al., 2022; Joffe et al., 2020b; Trantham-Davidson et al., 2014, 2017). To examine excitatory drive onto VIP-INs, we electrically isolated sEPSCs by voltage-clamping cells near the reversal potential for GABA_A receptors. Acute ethanol did not affect the amplitude or frequency of sEPSCs in VIP-INs (Fig. 5a and b). Similarly, we found no effect of IA ethanol or sex on sEPSC parameters (Fig. 5c and d). After abstinence from IA ethanol, we observed a significant interaction between IA ethanol and sex on sEPSC amplitude, but post hoc comparisons were not significant (Fig. 5e). No effects of abstinence or sex were

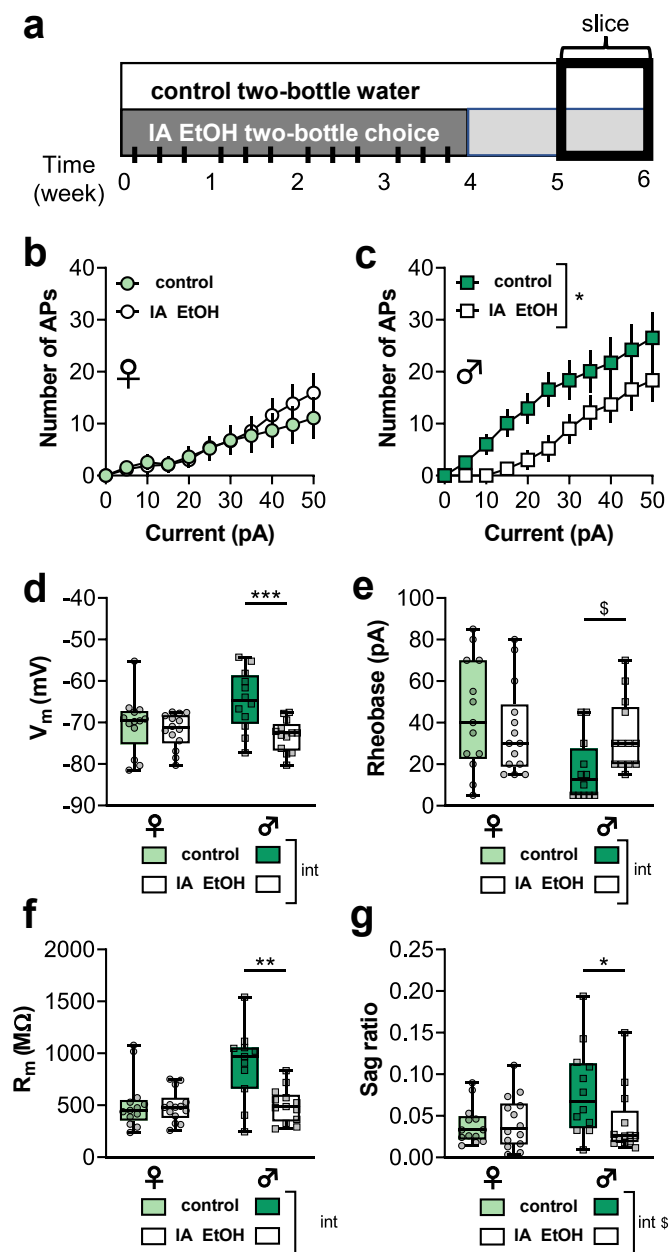


Fig. 4. Hypo-excitability of VIP-INs persists in male mice but not female mice. (a) Mice underwent 4 weeks of IA ethanol in their home cages, followed by 1–2 weeks of abstinence. Animals were sacrificed for electrophysiology 7–13 days after the final drinking session. (b) No difference in current-evoked spiking in VIP-INs between control and abstinent IA ethanol ♀ mice. (c) VIP-INs from abstinent IA ethanol ♂ mice displayed reduced current-evoked spiking relative to water controls (Two-way RM mixed-effects analysis: $F_{10,228} = 2.2$, $^*p < 0.03$ current $\times$ IA ethanol interaction). (d) In ♂ but not ♀ mice, VIP-INs displayed hyperpolarized V_m in abstinence from IA ethanol relative to water controls (Two-way ANOVA: $F_{1,48} = 4.9$, $p < 0.04$ sex $\times$ IA ethanol interaction; ***: $p < 0.001$ Bonferroni post test). (e) Abstinence from IA ethanol led to a trend increase in rheobase in VIP-INs from ♂ mice but not ♀ mice (Two-way ANOVA: $F_{1,48} = 4.5$, $p < 0.04$ sex $\times$ IA ethanol interaction; \$: $p < 0.1$ Bonferroni post test). (f) In ♂ but not ♀ mice, VIP-INs displayed reduced R_m in abstinence from IA ethanol relative to water controls (Two-way ANOVA: $F_{1,47} = 7.4$, $p < 0.01$ sex $\times$ IA ethanol interaction; ***: $p < 0.001$ Bonferroni post test). (g) Abstinence from IA ethanol decreased hyperpolarization sag in VIP-INs from ♂ mice but not ♀ mice (Two-way ANOVA: $F_{1,48} = 3.7$, $p < 0.06$ sex $\times$ IA ethanol interaction; *: $p < 0.05$ Bonferroni post test). $n/N = 12$ –14 cells from 3 mice per group.

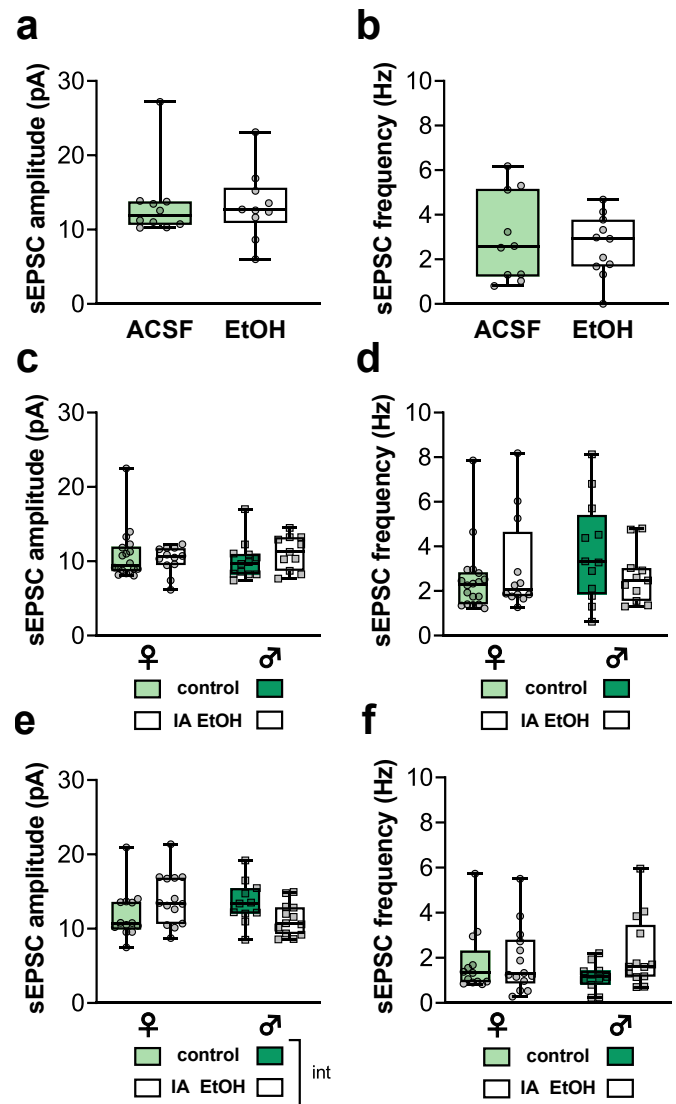


Fig. 5. Minimal effect of acute ethanol, IA ethanol, or abstinence on excitatory drive onto VIP-INs. Spontaneous excitatory postsynaptic currents (sEPSCs) were measured in voltage-clamp configuration. (a/b) Application of acute ethanol (20 mM) did not affect the amplitude or frequency of sEPSCs on VIP-INs. $n/N = 10$ cells from 5 mice (3♀/2♂) per group. (c/d) VIP-IN sEPSC amplitude and frequency were comparable between IA ethanol mice and water controls. $n/N = 13$ –20 cells from 3 to 5 mice per group. (e) An interaction between abstinence from IA ethanol and sex affected VIP-IN sEPSC amplitude (Two-way ANOVA: $F_{1,47} = 6.4$, $p < 0.02$ sex $\times$ IA ethanol interaction), but post hoc comparisons did not reach significance ($p > 0.14$). $n/N = 12$ –14 cells from 3 mice per group. (f) No effect of abstinence from IA ethanol or sex on VIP-IN sEPSC frequency.

observed regarding sEPSC frequency (Fig. 5f). These data indicate that ethanol and chronic drinking do not have a major impact on excitatory synaptic properties on VIP-INs and suggest that the observed changes to membrane physiology occur in a cell-autonomous manner.

3.6. Minimal effect of acute ethanol on VIP-IN excitability in socially isolated animals

During review of this manuscript, it was noted that the observed differences between the *ex vivo* and *in vivo* experiments could be related to differences in the housing status for these experiments. To address this concern, we revisited the acute ethanol application experiment in mice that had been housed in individual cages for 3 weeks in adulthood

(Fig. S5). In these experiments, we found no effect of acute ethanol (20 mM) on VIP-IN excitability. While VIP-IN cells from this experimental cohort appeared more excitable at baseline than cells from the initial experiments in group-house animals (Figs. 1 and 2); these experiments were conducted at different times and by different investigators, so potential effects of social isolation on baseline VIP-IN excitability remain speculative. Nonetheless, these findings suggest that housing status is a critical factor that regulates the effects of ethanol on VIP-IN excitability.

4. Discussion

The PFC is a key regulator of the acute and persistent effects of alcohol on brain function and behavior. PFC activity is dynamically modulated by VIP-INs, a unique type of neuron that inhibits other INs to enhance pyramidal cell activity (Audette et al., 2018; Lee et al., 2013; Pi et al., 2013; Williams and Holtmaat, 2019). To our knowledge, this series of studies represents the first published account of how ethanol and chronic drinking affect the function of PFC VIP-INs. Here, we found that ethanol acutely enhances the excitability of VIP-INs in brain slices. By contrast, following chronic voluntary drinking, VIP-INs displayed reduced spike-firing and greater rheobase relative to cells from water-only controls, consistent with a homeostatic reduction in excitability. Finally, we found that drinking-induced changes in VIP-INs persisted in male mice, but not female mice, over the course of 1–2 weeks of abstinence. Together, these studies identify unappreciated changes to PFC VIP-INs following acute ethanol and chronic drinking, paving the way for future studies to better understand the underlying mechanisms and to assess the relevance for problematic drinking behaviors.

Ethanol affects a wide variety of ion channels expressed in the central nervous system (Abraham et al., 2017; Harrison et al., 2017). In addition, a significant body of research indicates that neocortical and hippocampal INs are particularly sensitive to modest concentrations of ethanol (Carta et al., 2003; Harrison et al., 2017; Woodward and Pava, 2009; Yan et al., 2009, 2010). In these studies, we found that acute ethanol enhances the current-evoked spiking of PFC VIP-INs at a physiologically relevant concentration of 20 mM (~0.09 BEC) (Section 3.2). This effect could result from modulation of many targets, but we can begin to identify potential candidate molecules by evaluating the peripheral changes in membrane physiology. First, we observed that ethanol increased VIP-IN membrane resistance. As membrane resistance is inversely related to the number of functional ion channels on a neuronal membrane, it is likely that ethanol enhanced VIP-IN excitability by inhibiting one or more specific ion channels expressed by VIP-INs. Second, we found that ethanol decreased the rheobase and had minimal change on the resting membrane potential. These findings suggest that acute ethanol did not affect a tonic conductance, but instead acted on a voltage-gated channel. Taken together, this constellation of changes suggests that ethanol enhances VIP-IN activity by inhibiting a voltage-gated potassium current. Previous studies have found that ethanol inhibits KCNQ2 (or Kv7.2) currents at relevant concentrations (10–40 mM) in hippocampal pyramidal cells (Moore et al., 1990), in the ventral tegmental area (Koyama et al., 2007), and in heterologous expression systems (Cavaliere et al., 2012), supporting the hypothesis that KCNQ channels mediate ethanol's excitatory actions on PFC VIP-INs. This hypothesis could have exciting ramifications for potential breakthrough treatments, as the KCNQ channel potentiator, retigabine, attenuates drinking in rodent studies (Knapp et al., 2014; McGuier et al., 2016), and PFC *Kcnq5* expression negatively correlates with voluntary drinking (Rinker et al., 2017). While their ability to modulate INs in PFC has not been documented, KCNQ channels regulate the timing and summation of spikes within hippocampal INs (Lawrence et al., 2006), providing more support for this untested hypothesis. BK channels represent another class of voltage-gated potassium channel that could be involved in the present effects on VIP-INs. While initial studies showed that ethanol enhances BK (or KCa1.1) channel function (Dopico et al.,

1996), ethanol can also inhibit BK channels depending on post-translational modifications, intracellular calcium concentrations, and the co-expression of auxiliary subunits and associated proteins (Dopico et al., 2016). An important caveat to these conclusions is that we did not observe effects of ethanol on VIP-IN excitability in single-housed animals. One possible explanation is that social isolation stress occludes the effects of ethanol by increasing VIP-IN excitability on its own. Furthermore, all of the acute ethanol experiments in this manuscript were conducted with a single concentration (20 mM) designed to approximate the peak levels obtained during IA ethanol. Single-versus group-housed animals may exhibit differences in ethanol's potency on VIP-INs; thus, future experiments performing concentration-response curves are warranted.

While acute ethanol enhanced VIP-IN current-evoked spiking and decreased rheobase, we observed opposite effects following IA ethanol chronic drinking (Section 3.3). These findings support the notion that drinking precipitates a homeostatic change in VIP-IN membrane physiology, potentially driven by the same ionotropic species(s) inhibited by acute ethanol. We also observed a sex-dependent change in VIP-IN excitability after abstinence: VIP-IN hypo-excitability persisted in male mice, but the membrane physiology of VIP-INs from abstinent IA ethanol male mice was similar to respective controls (Section 3.4). One caveat to these findings is that the female control mice from the abstinence group appears to have reduced excitability relative to the female control groups in other studies. Based on this, it is possible that extended social isolation, or some other cohort-specific effect, may have prevented effects of ethanol from being detectable in this group of abstinent female mice. It is also possible that differences between the excitability of control VIP-INs in male mice in the abstinence group may have contributed to an emergent effect in that experiment. While experiments in this manuscript were not designed to directly assess how group-versus single-housing might affect VIP-IN physiology, future studies should take this into account. In addition, an unintended caveat to these findings is that female mice had slightly shorter abstinence times than males (7–8 days vs. 10–13 days) in these experiments. We do not think the difference in duration relates to the sex differences during abstinence for a few reasons. First, we observed no correlation between the length of abstinence and any aspect of VIP-IN physiology. Second, VIP-IN hypo-excitability was persistent in males and these animals displayed longer durations of abstinence. Finally, male mice displayed additional adaptations during abstinence that were never present in female mice: resting potential hyperpolarization and reduced sag. These two male-specific effects could both be explained by a reduction in HCN channel function. Previous studies have found that IA ethanol during adolescence can induce membrane hyperpolarization and reduce sag currents in PFC pyramidal cells (Salling et al., 2018). While these effects have not been observed when IA ethanol is initiated in adulthood (Joffe et al., 2021; Salling et al., 2018), it is possible that VIP-INs may express unique HCN channel subunits and/or auxiliary proteins that render them sensitive to ethanol's effects later in life. Support for this hypothesis can be found in previous studies in hippocampal INs. Ethanol has minimal effect on quiescent INs but increases spontaneous firing of active INs by facilitating HCN channel function (Yan et al., 2009, 2010). Thus, it is possible that chronic drinking may also induce a homeostatic change in HCN channel function in VIP-INs, at least in male mice. While we found no evidence for acute ethanol enhancing hyperpolarization sag or altering the resting potential in the present set of studies (Section 3.2), high cell-to-cell variation of membrane physiology in VIP-INs and/or the limited sensitivity of experiments in current-clamp configuration may have precluded the ability to detect subtle effects on HCN channel function. Future studies might be designed to test whether ethanol modulates VIP-IN HCN channels by performing directed studies in voltage-clamp configuration and/or by pharmacologically blocking other conductances (e.g. potassium currents) that might otherwise obfuscate subtle effects. Finally, future studies should assess whether the long-term effects of ethanol on VIP-INs might involve ethanol's ability to

potentiate 5HT₃ receptor function (Lovinger, 1999; Lovinger and White, 1991). From a translational perspective, investigating whether 5HT₃ receptors are involved in ethanol's actions on PFC interneurons in mice is critical, as cortical INs in humans do not express the 5HT₃ receptor (Krienen et al., 2020). Overall, mechanistic studies describing how drinking reduces VIP-IN excitability will be an important area of future research towards the identification of potential novel treatment targets.

Previous studies from our laboratory and others have examined how ethanol exposure affects PFC IN subtypes derived from the medial ganglionic eminence (e.g., PV-INs and SST-INs) (Dao et al., 2020, 2021; Ferranti et al., 2022; Fish and Joffe, 2022; Hughes et al., 2020; Joffe et al., 2020b; Li et al., 2021; Trantham-Davidson et al., 2014, 2017). Previous studies from our lab found that IA ethanol decreased the excitability of PV-INs but not SST-INs (Joffe et al., 2020b); thus, at face value, PV-INs and VIP-INs may undergo some similar long-term adaptations following chronic drinking. By contrast, we and others have found pronounced changes in excitatory drive onto both PV-INs and SST-INs following IA ethanol (Crowley and Joffe, 2022; Dao et al., 2021). As the present studies found no evidence for changes in glutamatergic synaptic strength on VIP cells, there are clearly important differences in IN subtypes' responses to long-term ethanol exposure. While future work will need to incorporate additional head-to-head comparisons and additional models of alcohol exposure, this limited literature suggests major differences in how ethanol alters distinct elements of PFC microcircuits. A limited and premature interpretation is that PV and SST cells are more tightly linked with glutamatergic signaling and synaptic plasticity, whereas VIP cells may respond and adapt more via neuromodulatory elements and/or plasticity of intrinsic excitability.

One of the major and well-described functions of VIP-INs is to decrease spontaneous SST-IN activity and ultimately facilitate pyramidal cell activity (Audette et al., 2018; Lee et al., 2013; Pi et al., 2013; Williams and Holtmaat, 2019). Recent studies by Li et al. (2021) found that acute injections of 2–3 g/kg ethanol can dampen PFC SST cell activity in vivo. In those studies, the authors found that these doses led to BECs between 20 and 50 mM at the timepoints where SST cell activity was reduced. The current findings suggest that increased VIP-IN activity could contribute to this effect, as our *ex vivo* data in slices suggests VIP cell activity can be enhanced at comparable ethanol concentrations. In addition, Li et al. (2021) revealed that low doses of alcohol (0.5–1 g/kg) attained BECs lower than 20 mM and increased SST cell activity. It is tempting to speculate that differences in ethanol's potency on regulating SST-IN versus VIP-IN membrane properties mediate the inverted U relationship observed by Li et al. (2021). Future studies that directly (and perhaps simultaneously) compare SST-IN and VIP-IN activity are needed to test this hypothesis. In addition, there are many other PFC cell types that could contribute to ethanol's acute effects on the structure. To our knowledge, this is the first report of ethanol's effects on any IN subtype that arises from the caudal ganglionic eminence. These INs represent a broad and heterogeneous group of neurons that preferentially reside in the superficial cortical layers (i.e., layers 1–3) (Krienen et al., 2020; Lee et al., 2010; Schuman et al., 2019; Tasic et al., 2018). While they are most abundant in layers 2/3, VIP-INs are also present in layer 1, unlike PV-INs or SST-INs. Despite their importance in regulating cortical function, layer 1 INs have been tremendously understudied in biological psychiatry research, and there are many other subtypes to examine beyond VIP-INs. In fact, nearly 90% of INs in layer 1 do not express VIP (Schuman et al., 2019); examining how these IN subpopulations are affected by acute ethanol and chronic drinking represents a compelling direction for future research. VIP-INs can also be subdivided into further subtypes with important functional and molecular distinctions. At the highest level, VIP-INs can be dichotomized into generally non-overlapping subpopulations based on expression of cholecystokinin (CCK) or calretinin (CR) (Porter et al., 1998; Tasic et al., 2018). VIP/CR-INs are commonly bipolar cells that inhibit other interneurons (Fu et al., 2015; Lee et al., 2013; Melchitzky and Lewis, 2008; Pfeffer

et al., 2013; Pi et al., 2013; Tasic et al., 2018). By contrast, VIP/CCK co-expressing INs are a type of basket cell that broadly dampens the activity of pyramidal cells through asynchronous GABA release (Freund et al., 1986; Kawaguchi and Kubota, 1998; Kubota and Kawaguchi, 1997). VIP/CCK-INs are more likely to reside in deeper cortical layers. Because the cells in the current studies all resided layers 1–3, the current results most likely relate to the function of disinhibitory VIP/CR-INs. Nonetheless, considering their opposing effects on pyramidal cell activity, it would be worthwhile for future studies to compare effects on VIP/CR-INs vs VIP/CCK-INs. Finally, a significant subgroup of VIP-INs co-expresses choline acetyltransferase (ChAT) and the other requisite molecules for acetylcholine neurotransmission (Eckstein and Baughman, 1984; Tasic et al., 2018). Recent functional studies have confirmed that VIP/ChAT-INs can directly excite other PFC cells via nicotinic receptors (Obermayer et al., 2019; von Engelhardt et al., 2007), thus they comprise an additional subpopulation that merits further study.

While significantly less is known relative to other IN subtypes, studies are beginning to describe how PFC VIP-INs regulate motivated and affective behaviors. The activity of VIP-INs is highly correlated with an animal's state of arousal (García-Junco-Clemente et al., 2017; Munoz et al., 2017), suggesting these cells may enhance the gain of salient information through cortical networks. Consistent with this idea, recent work has described how VIP-INs are recruited by reinforcing and punishing stimuli throughout all neocortical areas (Szadai et al., 2022). VIP-INs have been implicated in emotional behavior (Lee et al., 2019; Mossner et al., 2020), including aversiveness in response to neuropathic pain (Li et al., 2022). Based on this, it is tempting to speculate that changes in VIP-IN activity following IA ethanol are involved in affective disturbances and in heightened sensitivity to pain observed in many individuals with AUD. It will also be important to assess whether VIP-INs in other cortical areas respond to ethanol and adapt following chronic drinking. VIP-INs in somatosensory cortex regulate sensation, and these cells might be a substrate through which alcohol acutely alters vision, touch, and/or hearing. Therefore, future studies should certainly assess the functional relevance of alcohol's effects on VIP-INs in multiple cortical areas.

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Shannon M. Thompson: Validation, Investigation, Writing – review & editing. **Carly B. Fabian:** Validation, Investigation, Writing – review & editing. **Anthony S. Ferranti:** Investigation, Writing – review & editing. **Max E. Joffe:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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